

**Developmental regulation of adipose tissue growth through hyperplasia
and hypertrophy in the embryonic Leghorn and broiler**

Thesis

Presented in Partial Fulfillment of the Requirements for Undergraduate Research Distinction

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ABSTRACT

The United States is a world leader in poultry production, which drives producers to strive for better performance and muscle growth each year. Reducing accretion of adipose tissue is another important factor for poultry producers because this allows more nutrients to be directed towards muscle growth, but the impact of embryonic adipose growth on post-hatch development has not been fully understood. The purpose of this study was to investigate total DNA mass, morphological characteristics, differentiation markers, and triglyceride (TG) breakdown factors of embryonic adipose tissue, and their relation to hyperplastic and hypertrophic growth within layers (Leghorn) and meat-type (broiler) chickens. After 12 d incubation (E12), broiler embryonic weight was significantly higher than Leghorn, and this trend continued throughout incubation and post-hatch ($P < 0.05$). Neck and leg fat pad weights between the two breeds did not differ at each time point. Between E12 and E14, a remarkable increase in total DNA mass was observed in both Leghorn and broiler ($P < 0.05$), indicating a high potential for hyperplastic growth during this time. Histological analysis revealed clusters of preadipocytes at E12; however, the majority of these cells differentiated at E14 and continued to expand until time of hatch. Adipocyte sizes between both breeds did not generally differ even though broilers are known to have larger adipocytes post-hatch. Fatty acid-binding protein 4 expression levels in Leghorn and broiler continued to rise with each time point, which was paralleled with the expansion of mature adipocytes. Adipose triglyceride lipase was highly expressed at E20 and D0 to mobilize TG degradation for energy during hatching. Thus, embryonic chicken adipose tissues were found to develop by hyperplastic mechanisms followed by hypertrophy. At embryonic stages and early post-hatch, layer and meat-type chicken adipose growth does not differ, which suggests breed differences occur post-hatch.

Key words

embryonic adipose, total DNA mass, adipocyte hyperplasia, adipocyte hypertrophy, differentiation

INTRODUCTION

The United States is one of the leading producers and exporters of poultry products in the world, which is the reason why poultry producers are constantly searching for ways to maximize egg production in layers and muscle accretion in broilers (USDA, 2012). Genetic selection for performance and breast yield has become the main focus of the poultry industry (Abdullah et al., 2010). However, minimizing adipose accumulation has surfaced as a new target in the poultry industry due to the fact that small gains in feed conversion result in high production gains (Sizemore and Siegel, 1993). Broilers are known to have a higher adipose weight compared to layers, such as Leghorns, because their accelerated growth rates are accompanied by more muscle growth, but also more fat accretion (Emmerson, 1997). Insufficient information has been collected regarding embryonic adipose growth in both Leghorn and broiler to determine whether adipose development patterns differ at this stage and have an impact on post-hatch fat accretion.

Once the preadipocyte pathway is determined for a mesenchymal stem cell, maturation and growth is carried out by coordinated actions and signals. Hyperplasia, an increase in cell number, and hypertrophy, an increase in cell size, are the two mechanisms by which embryonic adipose tissue grows. In embryonic adipose development, hyperplastic growth of preadipocytes dominates, and hypertrophic growth is subsequent to fill the established cells with lipid (Jo et al., 2009). Adipokines and other signaling molecules, working at both the local and systemic level, promote or inhibit hyperplastic and hypertrophic mechanisms to regulate growth, along with

nutrient availability from the yolk (Kershaw and Flier, 2004; Moran, 2007). Preadipocyte differentiation and lipid gene regulation are controlled by certain key transcription factors, such as peroxisome proliferator-activated receptor γ (PPAR γ). One such gene regulated by PPAR γ is fatty acid binding protein 4 (FABP4), which mediates transport and metabolism of lipids within adipocytes (Damcott et al., 2004). As differentiation progresses, there is a dramatic increase in the expression of FABP4 (Billon et al., 2007). This differentiation marker is linked to hypertrophy by stimulating the transport of fatty acids for triglyceride (TG) synthesis to fill the adipocyte storage capacity (Sarruf et al., 2005).

Net accumulation of fat is the balance between TG synthesis and breakdown. TG breakdown is catalyzed by the rate-limiting enzyme, adipose triglyceride lipase (ATGL), also known as desnutrin, followed by actions of hormone sensitive lipase and monoacylglycerol lipase (Villena et al., 2004; Yang et al., 2013). ATGL hydrolyzes the first bond of a stored TG to release a fatty acid and diacylglycerol (Zimmermann et al., 2004). Two conserved domains were identified within adult avian species. A patatin domain exhibits lipase activity, while a hydrophobic domain binds lipid droplets (Lee et al., 2009). Relatively little, if any, information has been collected regarding ATGL mechanisms within the embryonic chicken.

Overall, hyperplasia and hypertrophy in embryonic chicken adipose tissue along with the influence of differentiation markers and TG breakdown factors have not been extensively studied, which leaves the impact on post-hatch performance and development to be unknown. Therefore, the aim of this study was to understand adipocyte growth mechanisms of hyperplasia and hypertrophy throughout embryonic incubation and compare the dynamics of adipose growth between layer-type and meat-type chickens. This will provide fundamental information on the

morphology, proliferation, and differentiation of adipocytes and potential similarities or differences during embryonic development of both breeds.

MATERIALS AND METHODS

Birds and Adipose Samples

Fertile Leghorn (layer-type) eggs were obtained from The Ohio State University poultry house (Lane Avenue), and fertile broiler (meat-type) eggs were obtained from Ridgway Hatcheries (La Rue, OH). Eggs were incubated and turned through a 90° arc every 2 h. At days 12, 14, 16, 18, and 20 of incubation (E12, E14, E16, E18, and E20), eggs were removed from the incubator and weighed, and 15 embryos from each breed were extracted with forceps and weighed. At day 1 post-hatch (D0), 15 chicks from each breed were humanely euthanized in accordance with the Institutional Animal Care and Use Committee at The Ohio State University and weighed. Fat pads on both sides of the neck and leg of each embryo or chick were excised and weighed (Figure 2). The adipose fat pads of the first five embryos or chicks collected from each time point were fixed in 10% neutral buffered formalin and designated for histology. The tissues of the second five embryos or chicks collected from each time point were immediately stored at –80°C for western blot analysis. The adipose tissues of the last five embryos or chicks were immediately stored at -80°C and utilized for determining total DNA concentration.

Visualization of Embryonic Fat Pads

Chicken embryos were removed from their eggs and washed with PBS several times. Then, embryos with feathers were further processed to remove the feathers by using forceps. Cleaned embryos were fixed in 10 % neutral buffered formalin for 48 h. The fixed embryos were

transferred into a 1% KOH solution for 16 - 48 h depending on the embryo size to visualize the fat pads by clearing skin and muscle tissues. Then, the embryos were transferred and kept in 100% glycerol until a picture was taken (Figure 2).

Total DNA Content

The adipose sample from the neck or leg was lysed in a volume of cell lysis buffer (CLS) (200mM NaCl, 50 mM Tris, 10 mM EDTA, 1% SDS; pH 8.0) appropriate for the weight of the fat pad (300 µl/ 10mg) in a 55°C water bath for 4 h with Proteinase K (1.5 µl/ 300 µl CLS) previously added. To remove residual protein, 300 µl of phenol-chloroform-isoamyl (PCI) was added to the lysates, vortexed, and centrifuged at 12,000x g for 2 min. Then, the supernatant was extracted, and 100 µl of 7.5 M ammonium acetate was added to the samples, vortexed, and centrifuged at 12,000x g for 10 min at 4°C. The supernatant was removed; and one volume of isopropyl alcohol was added, inverted about 50 times, and centrifuged at 12,000x g for 5 min at 4°C. The resulting pellet was washed with 70% ethanol and dried. DNA was resuspended in 100 µl of TE buffer (10mM Tris, 1 mM EDTA; pH 8.0) containing the RNase A (10 µg/ml) and stored at 4°C overnight. The next day, 200 µl of TE buffer was added with 300 µl of PCI, vortexed, and centrifuged at 12,000x g for 2 min. Then, the supernatant was extracted, and 70 µl of 7.5 M ammonium acetate was added to the samples, vortexed, and centrifuged at 12,000x g for 10 min at 4°C. The supernatant was removed; and one volume of isopropyl alcohol was added, inverted about 50 times, and centrifuged at 12,000x g for 5 min at 4°C. The resulting pellet was washed with 70% ethanol and dried. DNA was resuspended in 50 µl of TE buffer, and DNA concentration was measured with a Nanodrop spectrophotometer (Thermo Scientific,

Waltham, MA). Total DNA concentrations of the neck and leg fat pads were calculated with this data.

Adipocyte Histological Processing

Fat pads on each side of the neck and leg were fixed in 10% neutral buffered formalin, and then replaced with 70% ethanol. Tissues were dehydrated with 95% ethanol (2 x 40 min) and 100% ethanol (3 x 40 min), cleared in xylene (2 x 60 min), and embedded in paraffin. Microscope slides were prepared with three or four serial 8- μ m-thick slices using a microtome. Four slides were prepared for either the neck or leg of each animal. Slides were deparaffinized in xylene (3 x 4min) and hydrated with ethanol and dH₂O. Ethanol hydration consisted of 100% (2x 3 min), 95% (1 x 3 min), and 70% (1 x 3 min). Final hydration was performed in dH₂O (1 x 3 min). Slides were placed in Gill's hematoxylin (Vector Laboratories, Burlingame, CA) for 7 min and washed with running tap water for 7 min. Then, slides were dipped into acid ethanol (1% HCl in 95% ethanol) three times to remove residual hematoxylin. Slides were washed with dH₂O (2 x 2min), placed in eosin for 2 min, dehydrated with 95% ethanol (2 x 2min) and 100% ethanol (2 x 3min), cleared with xylene (2 x 3min), and a permanent cover slip was mounted with Permount Mounting Medium (Fisher Scientific, Waltham, MA). Stained slides were observed and imaged with AxioCam MRc 5 (Zeiss, Thornwood, NY), and ImageJ software (NIH ImageJ 1.47, <http://imagej.nih.gov/ij>) was used to determine cell size. Average adipose tissue cross-sectional area (CSA) was calculated by taking the area of a large portion of cells and dividing this by the total number of cells found within the area. At least 800 cells were evaluated per animal.

Western Blot Analysis

Protein lysates were isolated from neck fat using a lysis buffer (62.5 mM Tris, pH 6.8, and 1% SDS). Lysates were vortexed to ensure all cells were lysed, and the mixture was centrifuged at 13,000x g for 1 min. The supernatant containing the proteins was extracted and transferred to a clean tube. Then, 100 µl of 2x Laemmli buffer (62.5 mM Tris, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 12.5% glycerol, and 0.05% bromophenol blue) (Bio-Rad Laboratories, Hercules, CA) was added to 100 µl of the protein mixture. Gel percentage was chosen by target protein size, with smaller proteins being on higher percentage gels and larger proteins on lower percentage gels. Coomassie blue staining was used to visualize all proteins within the adipose samples. Proteins were wet-transferred to Immobilon Transfer membranes (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, plus 0.1% Tween 20) for 30 min at room temperature. Next, membranes were incubated for 1 h with a goat antibody against FABP4 (R&D systems, Minneapolis, MN) and with a rabbit antibody against human ATGL (1:1,000 dilution; Cell Signaling Technology, Danvers, MA). Membranes were washed 10 times in TBST, incubated in horseradish peroxidase-conjugated antibodies (1:2,500 dilution; Santa Cruz Biotechnology Inc.) for 1 h at room temperature, and washed another 10 times in TBST. Proteins were detected using ECL plus (Amersham Biosciences, Piscataway, NJ) and exposed to Biomax x-ray film (Amersham Biosciences).

Statistical Analysis

Descriptive statistics were calculated using the MEANS procedure of the Statistical Analysis System Institute (SAS Institute, 2009). A general linear model (GLM) was used to detect significant differences among chicken breeds and age groups. Significant differences of mean

values among chicken breeds and age groups were detected by the probability difference (PDIFF), and mean values were separated at the level of 5%. Results are presented as least squares means together with standard errors of the least square means.

RESULTS

Embryo, Body, and Adipose Weights

Embryo (EW) and body weights (BW) between Leghorn and broiler at each time point are presented in Figure 1. Flock ages and egg characteristics were not measured for this study, which may result in some variability in data depending on factors, such as hen age and yolk size. From E12 to E20, embryos of both breeds continually gained a significant amount of weight ($P < 0.05$), but E18 to E20 in Leghorn (19.9 vs. 39.3 g) and broiler (31.2 vs. 50.3 g) demonstrated the greatest increase in weight. Leghorn BW decreased slightly from E20 to D0, but was not significant (39.3 vs. 37.8 g). Broiler BW had a significant decrease from E20 to D0 (50.2 vs. 46.9 g, $P < 0.05$). When comparing Leghorn and broiler, there was no significant difference in EW at E12 (5.34 vs. 6.79 g, $P > 0.05$). Broiler EW and BW were significantly higher than Leghorn at E14 (11.0 vs. 14.9 g, $P < 0.05$) and at each subsequent time point ($P < 0.05$). A significant difference in EW occurred at E18 between Leghorn and broiler (19.9 vs. 31.2 g, $P < 0.05$).

Neck fat pad weights, leg fat pad weights, and neck and leg fat pad weight percentage relative to EW or BW are displayed in Figure 3. Neck and leg fat pad weights of Leghorn and broiler continually increased from E12 to E18 with a notable gain between E18 and E20 (0.124 vs. 0.243 g and 0.131 vs. 0.209 g, respectively, $P < 0.05$). Between E20 and D0, there was a significant drop in neck fat pad weight for Leghorn and broiler (0.243 vs. 0.196 g and 0.209 vs.

0.169 g, $P<0.05$) and in Leghorn leg fat pad weight (0.221 vs. 0.181g, $P<0.05$), which parallels with the trends in EW and BW. Comparing data between breeds, neck fat pad weights in Leghorn and broiler were similar until E20 and D0 when Leghorn was higher (0.243 vs. 0.209 g and 0.196 vs. 0.169 g, $P<0.05$), while leg fat pad weights varied at E18 and D0 when broiler was higher (0.141 vs. 0.170 g and 0.181 vs. 0.231 g, $P<0.05$). Leghorn and broiler fat weight percentage relative to EW or BW was not different at E12. Neck fat pad weight percentage relative to EW or BW after E12 was significantly lower in broiler due to their larger EW and BW. Leg fat pad weight percentage relative to EW or BW after E12 was lower in broiler until D0, which equaled Leghorn (0.48 vs. 0.49%).

Total DNA Content

Total DNA content within the neck fat pads and leg fat pads of Leghorn and broiler are shown in Figure 4. Neck DNA mass was approximately 4.0-fold higher in Leghorn from E12 to E14 and 3.6-fold higher in broiler from E12 to E14 (7.69 vs. 31.2 μg and 8.27 vs. 30.1 μg , $P<0.05$). This trend was also observed in leg DNA mass from E12 to E14 with a 4.4-fold increase in Leghorn and a 2.6-fold increase in broiler (8.24 vs. 36.6 μg and 12.9 vs. 33.9 μg , $P<0.05$). Leghorn DNA contents in both neck and leg fat pads increased until E18, which marked the time point when the levels achieved a steady state. The same trend was noticed in broiler DNA contents, but a slight increase was observed at D0 in neck and leg. Between Leghorn and broiler, DNA mass did not differ at any time point in the neck or leg.

Adipocyte Size and Characteristics

Histological images of E12 neck and leg fat pads in Leghorn and broiler are presented in Figure 5A. Clusters of nuclei demonstrated the groups of preadipocytes that would eventually differentiate into multilocular adipocytes under optimal circumstances. Several multilocular or small unilocular adipocytes were visible; therefore, the adipocyte size at E12 could not be measured. Figure 5B displays histological images of adipocytes in the neck and leg of both breeds at all of the older time points. A clear expansion of adipocyte size is evident from E14 to E20 in each breed, and a slight decline in size is noticeable from E20 to D0, which resembles the reduction in EW or BW and fat pad weight at this point. Adipose tissue CSA of neck and leg fat pads in both breeds are shown in Figure 5C and 5D, respectively. Similar to the histological images, there is a continuous increase in adipose tissue CSA from E12 to E20 and decline at D0 in the neck and leg fat pads of each breed. Adipose tissue CSA of Leghorn and broiler neck did not differ at E14, E18, and D0; however, adipose tissue CSA of Leghorn neck was larger at E16 (907 vs. 711 μm , $P<0.05$) and E20 (1433 vs. 1386 μm , $P<0.05$). Adipose tissue CSA of Leghorn leg was larger than broiler at E14 (566 vs. 463 μm , $P<0.05$), E16 (794 vs. 676 μm , $P<0.05$), and E18 (1058 vs. 973 μm , $P<0.05$), but Leghorn was smaller than broiler at D0 (1086 vs. 1137 μm , $P<0.05$).

Adipogenic and TG Breakdown Markers

Protein expression by western blot analysis of FABP4 in the mouse, chicken, cattle, and pig and adipose-specificity of FABP4 are shown in Figure 6A. Although the FABP4 signal was not strong for the chicken compared to other species, it was still detectable within the adipose tissue, validating usefulness of this FABP4 antibody for studying chicken FABP4 proteins. Similar to mouse, bovine and porcine FABP4 proteins (Shin et al., 2009), the adipose-specific nature of

FABP4 was displayed in the abdominal fat (AF) and subcutaneous fat (SQ) of the broiler, while no expression was detected within the kidney, liver, lung, heart, pectoralis muscle, or tibialis muscle. Figure 6B displays FABP4 and ATGL expression levels in Leghorn and broiler from E12 to D0. FABP4 was expressed at increasing levels with each time point in Leghorn and broiler, correlating to growing numbers of mature adipocytes in the animal. At E20 and D0, dramatic increases in expression of ATGL were exhibited in both breeds. This is the point when lipids must be mobilized to provide energy for the hatching process. Leghorn and broiler exhibited similar expression patterns for both FABP4 and ATGL.

DISCUSSION

Meat-type chicken embryonic growth rates begin to accelerate past layer-types around the middle of embryonic development. This early rapid growth may be attributed to a larger yolk mass within the meat-type egg, which corresponds to higher nutrient availability (Ho et al., 2010). Yet, the pathways and targets of these nutrients have not been clarified. Fat pads in both breeds can be seen with the naked eye starting at E9. In the current study, EW gain in Leghorn and broiler was constant until a considerable leap in EW occurred in both breeds from E18 to E20, which is attributed to incorporation of the yolk into the embryo. Between E20 and D0 in both breeds, BW and fat pad weights declined, which due to increased energy expenditure during hatching. Chickens have a high demand for energy during the hatching process; therefore, ATGL becomes functional to mobilize lipid within the adipose tissue, and a decline in BW and fat pad weight takes place (Lee et al., 2009). Free fatty acids (FFAs) released by the adipocyte are shuttled to the liver. The FFAs that are not re-esterified are utilized as a primary energy source for fasting periods, such as directly after hatching (Sun et al., 2011). In the poultry industry, feed

is often withheld following hatching because of sexing, vaccination administration, and transportation. This period causes weight loss in the chicks as they utilize lipids from the yolk or adipose tissue. Thus, up-regulation of ATGL occurs in response to this feed restriction to promote lipolysis and release fatty acids for energy (Lee et al., 2009). When assessing differences between breeds, broiler EW was significantly higher at E14 and each subsequent time point ($P<0.05$). The gap in EW widened as both breeds reached E18 with broiler exceeding Leghorn, possibly because of larger muscle accretion from genetic selection, and this trend did not shrink after hatch. Interestingly, neck and leg fat pad weights did not greatly differ between Leghorns and broilers, and broilers had lighter neck fat pad weights at E20 and D0. When the fat pad weights were compared with total EW or BW, Leghorns usually had a higher fat percentage within the body. Due to extensive genetic selection within broiler strains, accelerated growth of the pectoralis muscle is noted even during embryonic stages, which can offset the adipose to BW ratio (Emmerson, 1997).

Hyperplasia and preadipocyte differentiation are some of the first mechanisms observed during adipose tissue development (Jo et al., 2009). This increase in preadipocyte number ultimately lays the foundation for differentiation to mature adipocytes and accumulation of lipid. As the number of cells continues to rise, total DNA mass is directly proportional to the number of cells within the tissue because each cell contains the same amount of DNA, and therefore indicates points of hyperplastic growth (Guo et al., 2011). In Leghorn and broiler, a significant increase ($P<0.05$) in total DNA content was observed from E12 to E14 in both neck and leg fat pads. Increase in total DNA mass between E12 and E14 could illustrate induced hyperplasia followed by a slowing of cell number expansion as embryos reach E18. Leghorn DNA mass plateaued after E18, but broiler DNA mass had a minor increase at D0, which implied another

small expansion of cell number. Substantial gatherings of preadipocytes were found in E12 Leghorn and broiler adipose tissue, indicating low levels of differentiation. Mature adipocytes with smaller pockets of preadipocytes were noticeable as the embryos grew to E14. During this stage of incubation, the adipose tissue in both breeds is undergoing an expansion of cell number, and preadipocytes are rapidly differentiating into mature, lipid-containing adipocytes (Speake et al., 1996).

As differentiation of preadipocytes progresses, cells first become multilocular with several small vesicles of lipid, and then they expand and join to form one lipid droplet, termed unilocular. The transition from multilocular to unilocular adipocytes occurs at varying times across species. Hausman and Kauffman (1986) discovered that fetal to day 1 postnatal pigs possessed multilocular adipocytes and did not develop any unilocular adipocytes until day 3. At day 9, the majority of adipocytes were unilocular, while multilocular cells were found sparingly. When examining adipose tissue of day 1 old calves, unilocular adipocytes are present with relatively no multilocular adipocytes (Smith et al., 2004), demonstrating earlier maturation of adipocytes. Chickens, as well as other avian species, require an adequate amount of lipid transferred from the yolk to hatch at the end of incubation and continue heat generation (Moran, 2007). At E12 in Leghorn and broiler, almost all of the adipocytes were multilocular with some unilocular cells interspersed. Once the embryo reached E14, most of the adipocytes were unilocular with some multilocular cells visible, and then, multilocular cells were nearly undetectable at E16. Therefore, adipocytes in the chicken undergo rapid maturation to prepare for hatching at the end of incubation.

As the adipocyte matures, it begins to expand with incoming lipid, which is known as hypertrophic growth (Jo et al., 2009). Adipocytes are unique in that they have gigantic storage

capacities and can swell as much as 100% of their original sizes (Johnson and Francendese, 1985). After day 12 of incubation, TGs rapidly begin to deposit within subcutaneous adipose tissue in chicken embryos (Speake et al., 1996). FABP4 binds and carries long-chain fatty acids to maintain accumulation of TGs within maturing adipocytes (Shi et al., 2010). A continuous gain in adipocyte size was noted from E14 to E20 in Leghorn and broiler. Then, adipocyte size slightly receded at D0 due to previously explained mechanisms of ATGL. Jo et al. (2009) revealed a strong positive correlation between fat pad mass and volume-weighted mean cell size in mice, indicating that hypertrophy is the main contributor to increases in adipose tissue mass. Although fat pad weight and DNA were the same between Leghorn and broiler at E16, CSA was significantly higher in Leghorn than broiler, especially in the neck. This may be attributed to faster maturation of adipocytes in the Leghorn accompanied by many small, immeasurable preadipocytes that still contribute to DNA mass and fat pad weight if present in high enough quantities. Contrarily, broiler adipocytes may possess a slow, steady growth rate with more differentiated preadipocytes, but the same number of adipocytes is present in the fat pad.

In summary, total DNA contents accompanied by increases in adipocyte size exemplify the two mechanisms of hyperplasia and hypertrophy that contribute to adipose mass. To our knowledge, this is the first study investigating the fundamental aspects of adipose growth and development in chicken embryos. In conclusion, it was determined that, although meat-type chickens possess larger EW, weight and development of embryonic adipose tissue is not noticeably different between meat-type and layer-type chickens. Therefore, post-hatch events are most likely the reason for differences in adipose weights between the two breeds. More information on molecular pathways of embryonic chicken adipose tissue growth must be

collected along with investigation of post-hatch development differences of layers and meat-type chickens to apply this knowledge for reducing fat accretion in the poultry industry.

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FIGURES

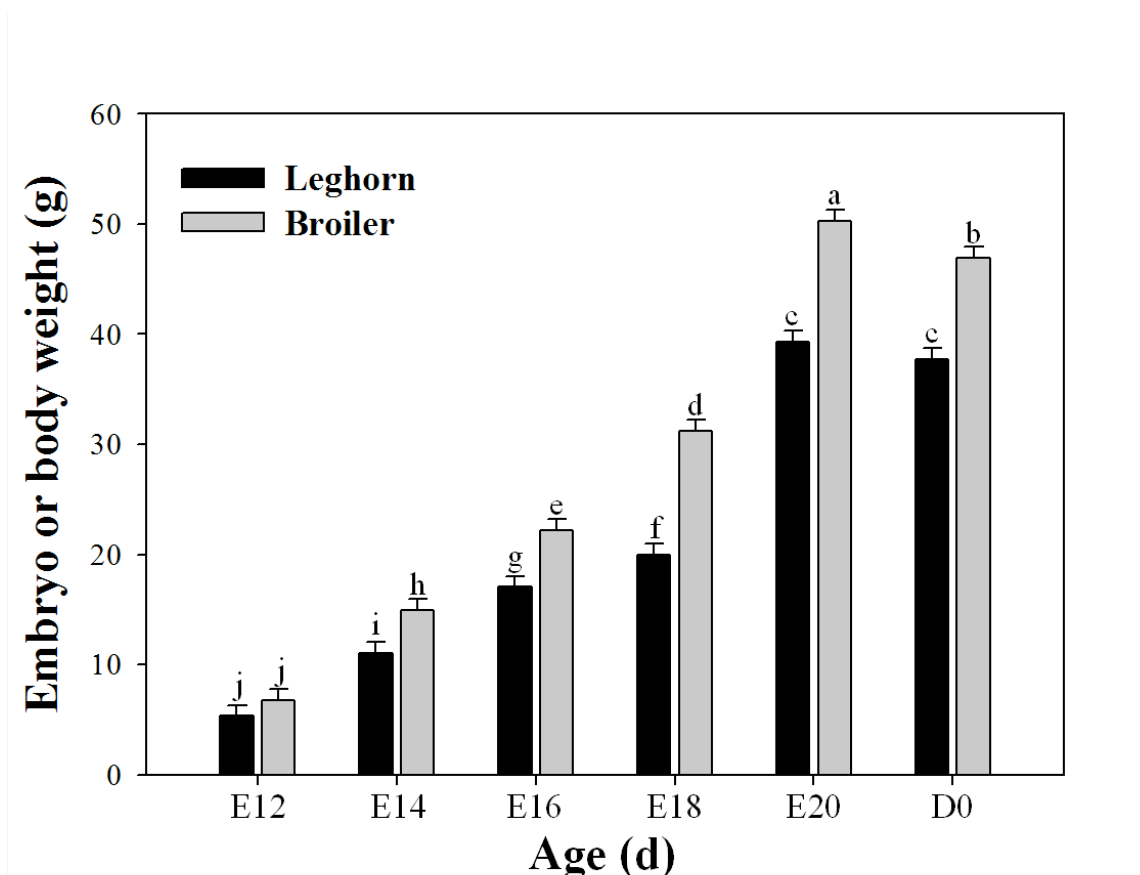


Figure 1.

Comparison of embryo weight (EW) from embryonic day 12 (E12) to embryonic day 20 (E20) and body weight (BW) at day 1 post-hatch (D0) between Leghorn and broiler (n = 15 per time point per group). Bars represent mean \pm SEM. Significance ($P < 0.05$) indicated by different letters.

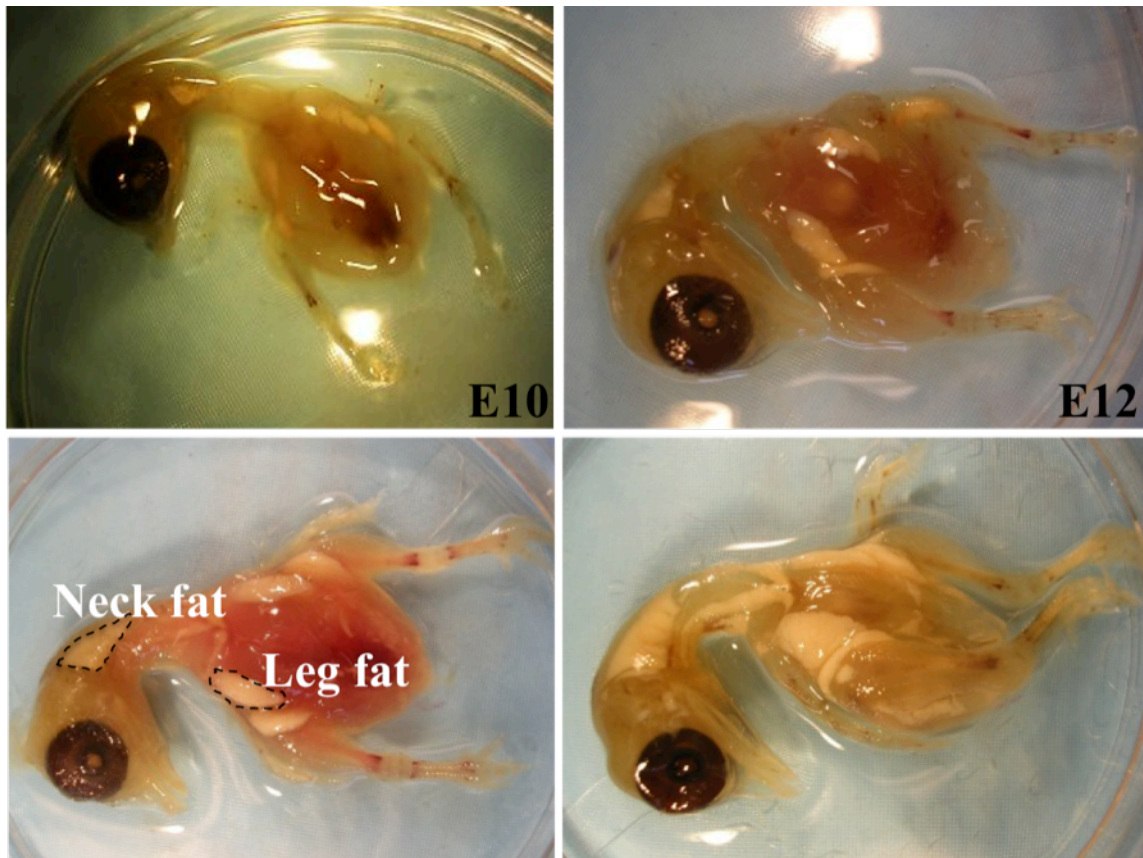


Figure 2.

Leghorn embryo fat pad visualization with 1% KOH at days 10, 12, 14, and 16 of incubation.

The left neck fat pad and right leg fat pad are indicated by dashed lines on E14 embryo. Images are not to scale.

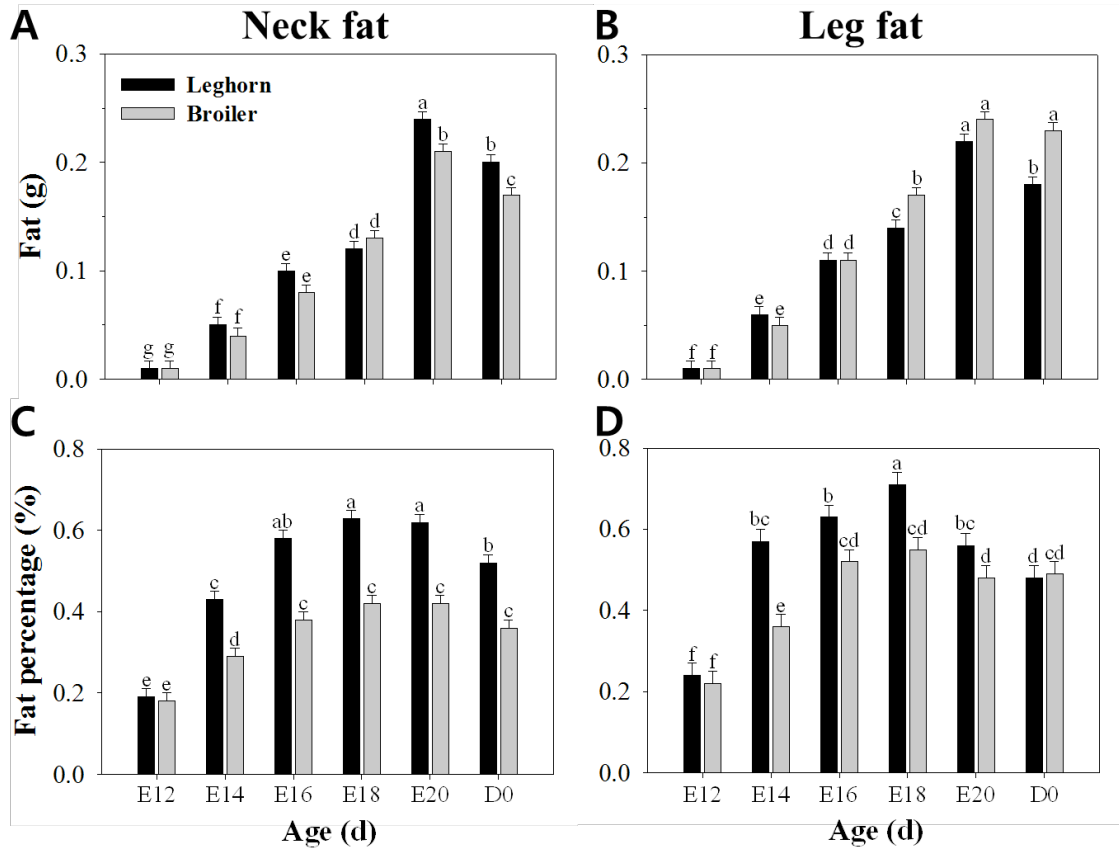


Figure 3.

Comparison of fat pad weights (A) in the neck (B) and in the leg (C) with neck fat percentage (D) and leg fat percentage relative to EW or BW between Leghorn and broiler (n = 15 per time point per group). Bars represent mean \pm SEM. Significance ($P < 0.05$) indicated by different letters.

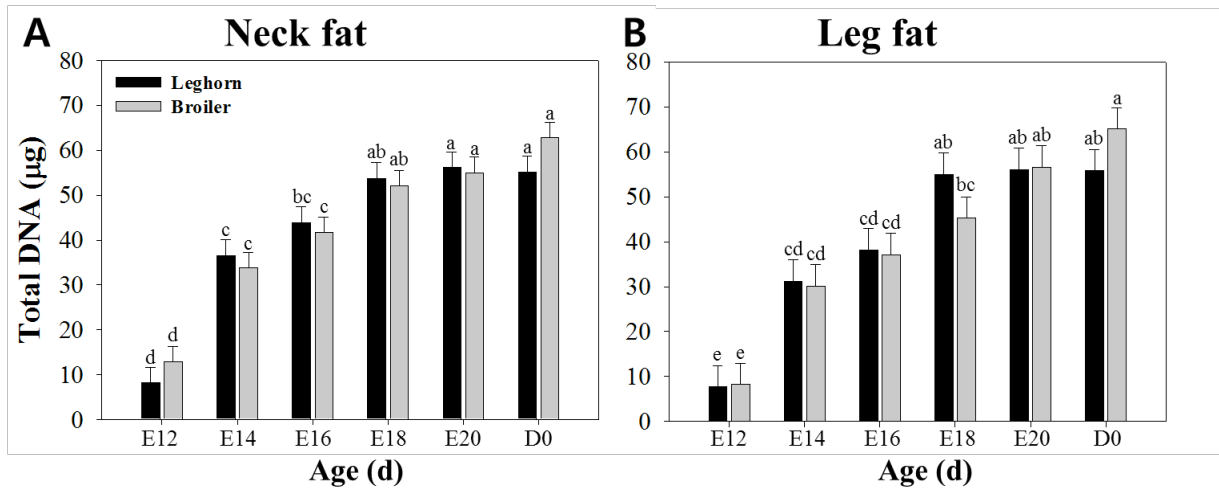
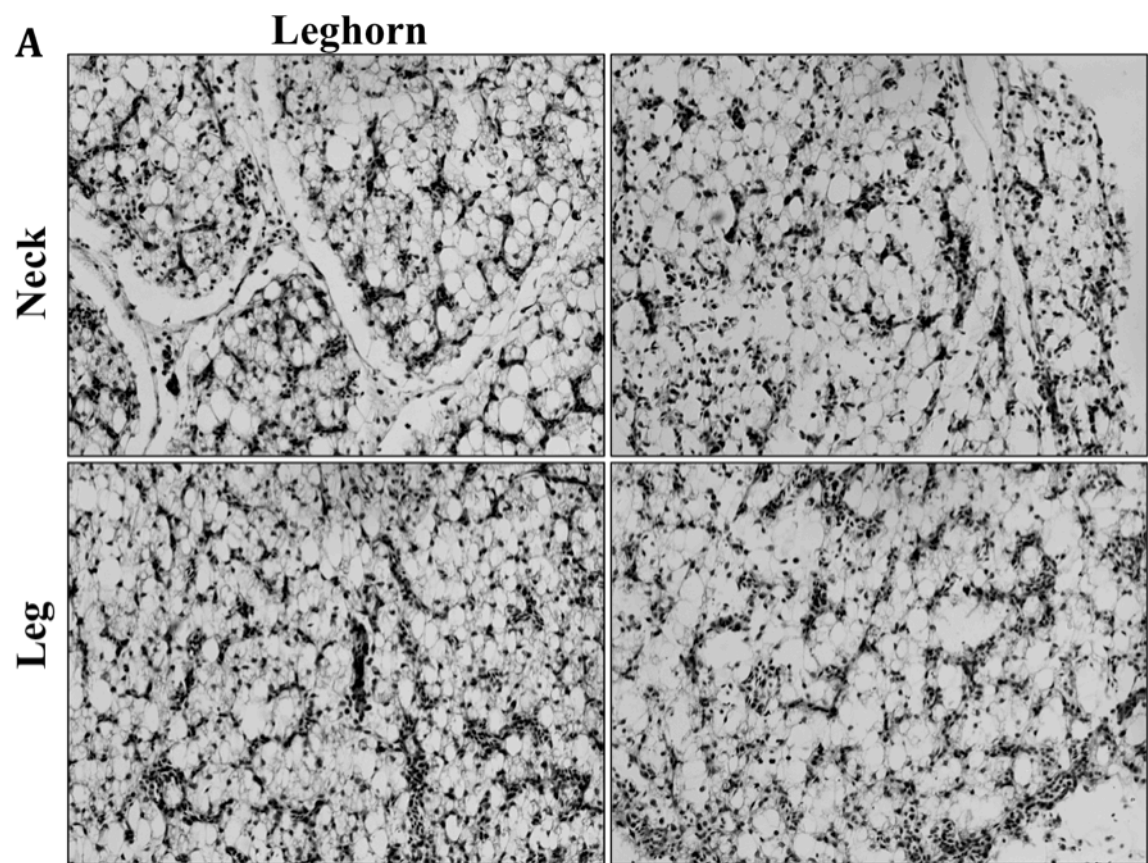


Figure 4.

Comparison of total DNA content (A) within the neck fat pad (B) and the leg fat pad between Leghorn and broiler (n = 5 per group). Bars represent mean \pm SEM. Significance ($P < 0.05$) indicated by different letters.



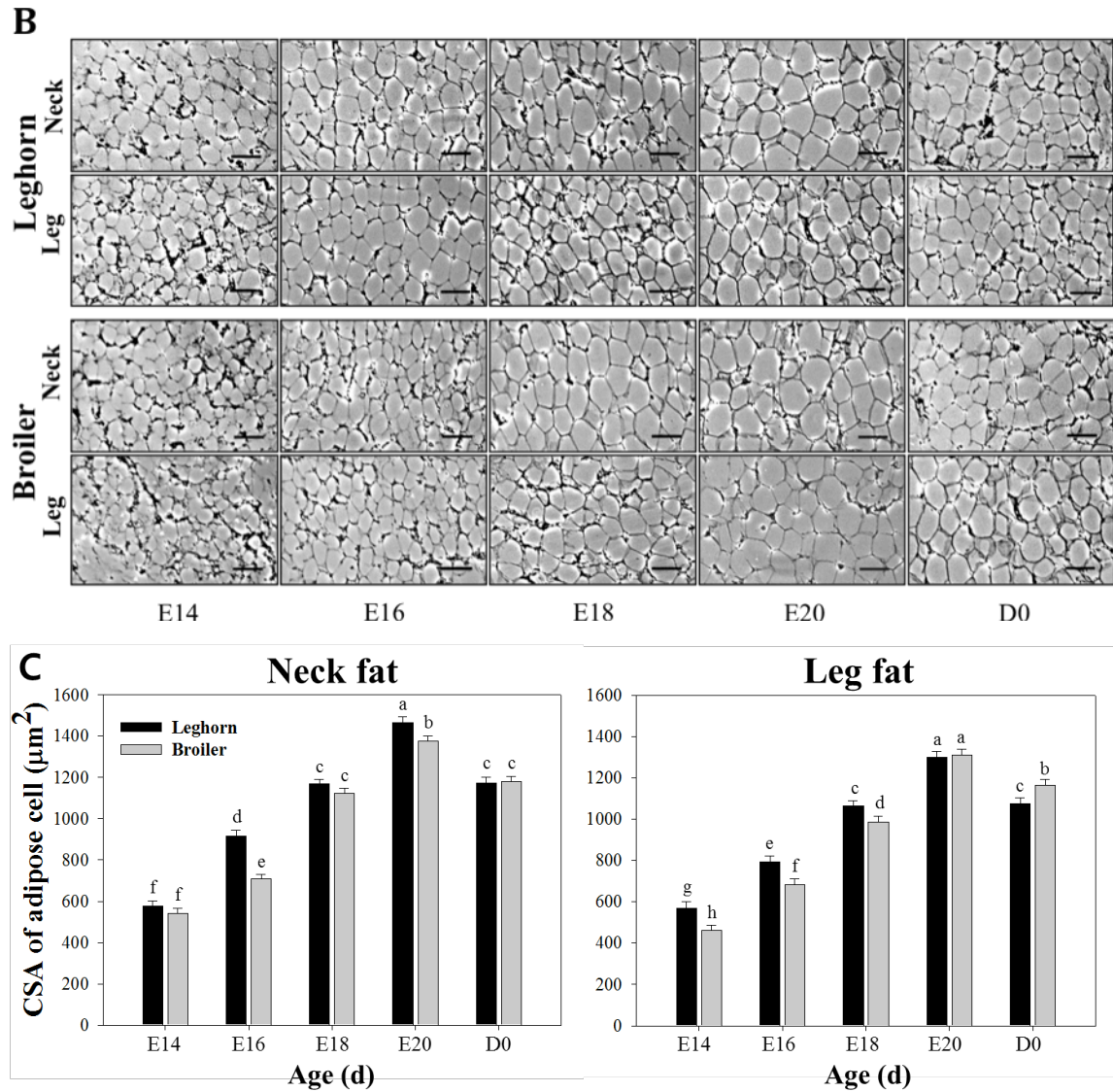


Figure 5.

Histological images of (A) embryonic day 12 (E12) neck and leg adipose tissue at 200x (B) and subsequent time points (E14-D0) for Leghorn and broiler at 100x. Scale bar = 50 μm . Further comparison of cross-sectional area (CSA) (C) of the neck fat pad and leg fat pad in Leghorn and broiler (n = 5 per group). Bars represent mean \pm SEM. Significance ($P < 0.05$) indicated by different letters.

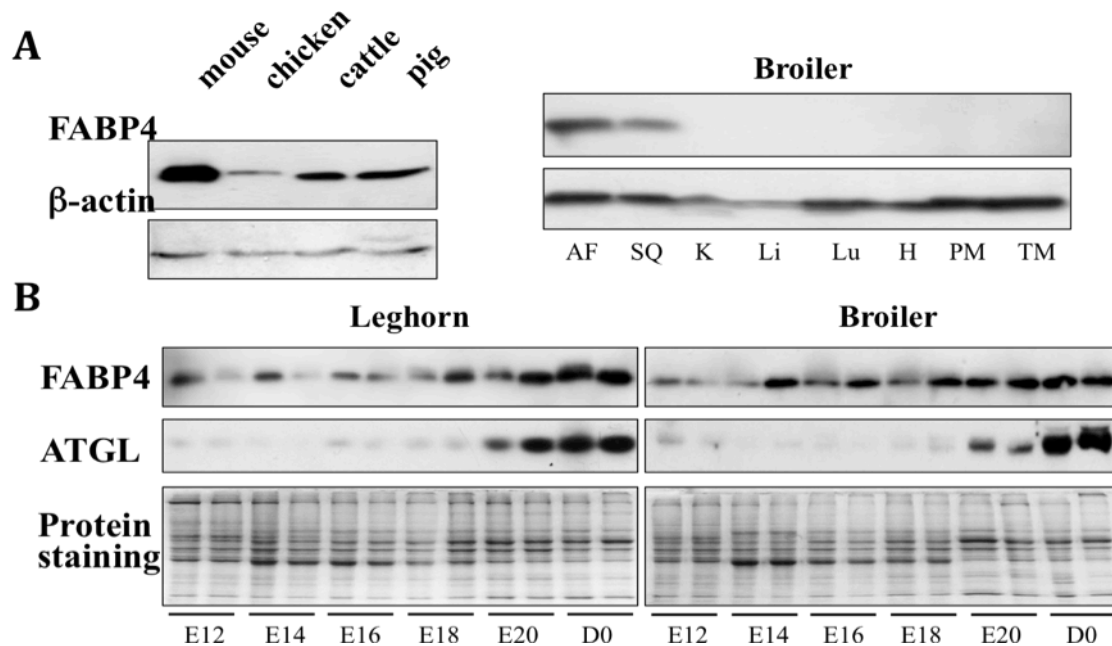


Figure 6.

Western blot analysis for (A) FABP4 expression demonstrated in the mouse, chicken, cattle, and pig with normalization to β -actin and adipose-specificity shown in the broiler compared to other tissues. The two fat samples depicted are abdominal fat (AF) and subcutaneous fat (SQ). No expression of FABP4 was found in the kidney, liver, lung, heart, pectoralis muscle, or tibialis muscle. (B) Leghorn and broiler expression levels of FABP4 and ATGL from embryonic day 12 (E12) to day 1 post-hatch (D0). Coomassie blue protein staining was used as a standard instead of using cytoskeletal proteins, including β -actin or α -tubulin, that showed variation in the amount of these proteins during embryonic adipose development that is usually accompanied with dynamic changes in cytoskeleton structures.